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Magnetic resonance spectroscopy for the determination of renal metabolic rate in vivo

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Magnetic resonance spectroscopy for the determination of renal metabolic rate in vivo. The method of magnetic resonance spectroscopy has been validated and applied to the determination of renal metabolic rate in vivo. Using an indwelling detector coil, ^{31}P NMR spectra from one kidney of anesthetized rats were quantified. The concentration of ATP was the same as that determined enzymatically, but both ADP and Pi were substantially lower. Only 25% of renal Pi and virtually none of the ADP were detected by NMR. The remainder is assumed to be bound to proteins. These concentrations of metabolites contributed to a significantly increased phosphorylation potential, which in turn should increase the free energy of hydrolysis of ATP. Saturation transfer, a non-destructive magnetic technique for the measurement of chemical exchange, was readily able to detect synthesis of ATP from ADP and inorganic phosphate. The rate of ATP synthesis determined was comparable to that determined in parallel studies of renal oxygen consumption. An ATP:O ratio of approximately 2 was found, indicating that fatty acid is the preferred fuel of respiration of the rat kidney in vivo.

Nuclear magnetic resonance spectroscopy applied to the non-destructive detection and assay of phosphorus metabolites (^{31} phosphorus NMR) has found a place in studies of renal metabolism [1–4]. The earliest studies successfully explored the use of ^{31}P NMR to measure intracellular pH; significant changes in intra-renal pH were observed in total renal ischemia [1], in metabolic acidosis [2], in respiratory acidosis [3] and in a number of models of acute renal failure [4]. Depletion of renal ATP by ischemia has also been documented [1, 4] but in these early studies, only semi-quantitative results were obtained. This is a serious limitation of many biological studies with NMR, where rapid pulsing has been used to achieve sufficient signal at the expense of significant saturation of the NMR response. When suitable pulse intervals are employed, quantitative data can be obtained. This then permits validation of the NMR studies by direct comparison with accepted methods. We have recently demonstrated that in the kidney the concentration of ATP ([ATP]) can be determined with reasonable precision, and is the same by NMR and by enzymatic assay. However, ADP and inorganic phosphate (P_i) are both significantly bound within the renal cell. This results in a very much lower tissue concentration for these two metabolites when measured with NMR [5, 6]. It is likely that NMR will assume particular significance as a means of measuring the metabolically active pool of intermediates, with far reaching consequences for our understanding of important processes, such as oxidative phosphorylation [5], sodium transport [5] and phosphate transport [7]. Because these observations were made in an isolated perfused kidney model, it was important to confirm that they also apply to the intact kidney in vivo. The present studies quantify adenine nucleotides, intra-renal pH and P_i in the rat kidney in vivo for comparison with the perfused kidney, and with standard enzymatic assays of the kidney freeze–clamped in vivo.

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Determining renal metabolic rate

A further important measurement made with NMR in the isolated kidney was the metabolic rate, or the rate of ATP synthesis from ADP and P_i. This was achieved using the technique of saturation transfer NMR (STNMR) [5]. Introduced by Brown, Ugurbil and Shulman (8) into biology for the determination of the rate of ATP formation in E. coli, this method depends upon the use of a selective pulse of radiofrequency which saturates the magnetic signal from one reactant, while observing the amplitude of the magnetic signal in the second reactant. The technique applies to reactions which are readily reversible. It was developed by Forsen and Hoffman [9] from studies of first order reactions in solution. Criticisms of the technique applied in an intact organ depend upon theoretical existence of multiple pools of metabolites, poorly reversible reactions, and other fates of the reactants different from the single metabolic interconversion under consideration. These objections can only be answered by experiment. In the recent study by Freeman et al [5], it was demonstrated that significant exchange occurred between the γ-phosphorus of ATP and P_i in the intact, perfused kidney, and that the extent of transfer was compatible with a renal metabolic rate, which was matched by the oxygen consumption measured at the same time. A most important assumption thereby tested experimentally was that [P_i] quantified by NMR was that fraction of the total P_i pool which was involved in oxidative phosphorylation. Furthermore, the result suggested that alternative reactions of γ-phosphorus of ATP and of P_i were of minor importance compared with oxidative phosphorylation itself.

If saturation transfer NMR is to be of value in intact animals, and ultimately in monitoring the rate of renal metabolism in

man, these studies in the isolated kidney need to be confirmed for the in vivo kidney. The present study was undertaken for that purpose. Quantitative ^{31}P NMR spectra of rat kidneys were obtained, using an indwelling detector coil, around the kidney of an anesthetized rat. Saturation transfer was determined during saturation of the γ-phosphorus of ATP, and renal ATP synthesis thereby determined. Oxygen consumption was determined in other rats by direct arteriovenous oxygen difference across the kidney, and renal blood flow measured from microsphere injection.

A preliminary account of these experiments has been briefly reported previously [10, 11].

Methods

Animals

Well fed, male Wistar rats, 250 to 350 g body wt, were used in all in vivo studies. Animals between 300 and 400 g were used in kidney perfusion experiments. Because in vivo, oxygen consumption could not readily be determined at the same time as the NMR study, different groups of animals from the same source were used for the determination of renal blood flow and oxygen consumption. The experimental procedure used differed slightly, in respect of anesthetic agent and the posture of the animals, but were optimal for the chosen measurement in each case.

^{31}P NMR studies in isolated perfused rat kidney

The details of the kidney perfusion procedure [2] and of methods used to collect ^{31}P NMR and STNMR spectra from the perfused kidney have been given previously [5]. Kidneys from twelve rats were perfused for between one and four hours for comparison with in vivo results. The kidney, surrounded by a solenoid coil which served both to generate and receive radiofrequency NMR signals, was maintained by perfusion through the renal artery. Perfusate was a blood–free solution containing bovine serum albumin (7.5 g/100 ml) in phosphate–free Krebs–Henseleit saline. To this were added glucose (5 mM) and an amino acid mixture designed to maintain optimum renal transport function [12]. Glomerular filtration rate (^{14}4C-inulin clearance), Na^+ transport and oxygen consumption were the criteria used to confirm adequacy of renal function. At the same time, quantitative ^{31}P NMR determinations were carried out [5]. The results were compared with those of chemical and enzymatic assay of ATP, ADP, AMP, and P_i in perchloric acid extracts of the same kidneys freeze–clamped after perfusion.

^{31}P NMR studies in vivo

The technique for obtaining continuous NRM signals from the kidney of rats in vivo was developed by Chan (D Phil Thesis, University of Oxford) and has been described earlier [4]. In brief, rats were anesthetized with Nembutal (5 mg per 100 g body wt by intraperitoneal injection). Anesthesia was maintained in the ensuing three to four hours by a mixture of 0.5-1% Halothane in nitrous oxide, oxygen flowing at a rate of 0.5 to 1.0 liters/min through a face mask. Animals could be maintained with a stable blood pressure for four to six hours by this means, sufficient for the conduct of saturation transfer experiments. Other anesthetic regimes were less satisfactory in this regard.

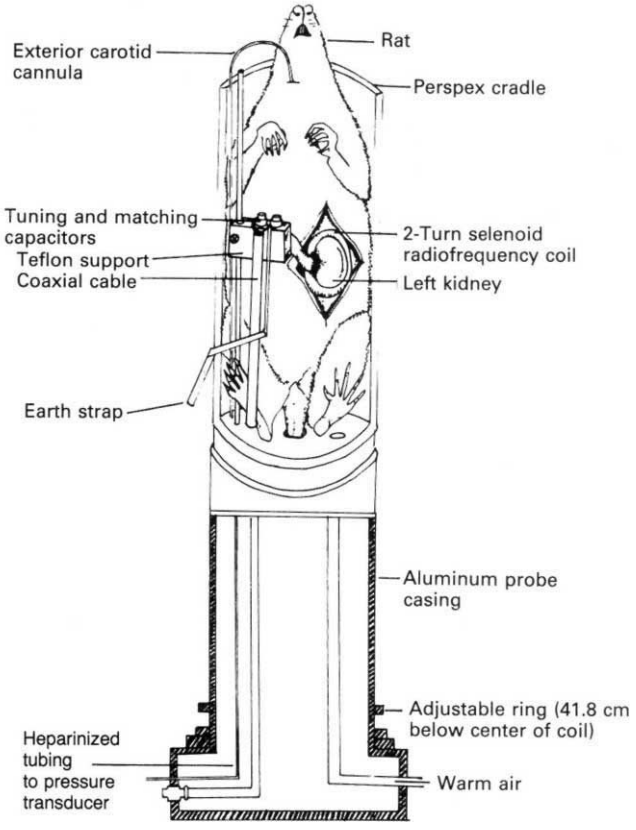

Fig. 1. *Animal cradle for in vivo ^{31}P NMR studies.* Tuning to ^{31}P and shimming with ^1H signals preceded three to four hr studies for saturation transfer NMR. Note that the cradle is not drawn exactly to scale.

A mid-line incision was made in the abdomen and the left kidney was freed from surrounding fat. With minimal disturbance of the renal pedicle, the kidney was placed within a coil. The coil consisted of two turns of copper wire, forming a solenoid of approximately 1 × 1.5 cm diameter. This was arranged to enclose the whole of the kidney in its long axis. The wire was insulated and in the gap between the turns a capillary of similar diameter and configuration contained the calibration standard.

^{31}P spectra of kidney are normally virtually free from PCr [5]. The coil was positioned around the kidney in such a way as to avoid receiving PCr signals from the adjacent abdominal muscle. This was achieved by careful positioning of the coil, and inter-position of gauze soaked in saline between kidney and the muscle wall. The position of the kidney and coil within the magnetic field of the spectrometer was standardized by adjustment of the position of the animal in the cradle (Fig. 1). For calibration of quantitative NMR spectra, the coil was modified by addition of a tube of 4.5 μmoles methylene diphosphonate between the turns. The abdomen was closed, and the animal mounted in a cradle designed to be inserted in the vertical position head up in the bore of the magnet. When positioned, the coil, with the kidney enclosed, lay in the homogeneous, sensitive volume of the spectrometer. The body temperature of

the animal was maintained at 36°C ± 0.5°C by means of a stream of warmed air passing up the bore of the spectrometer.

Calibration of standard

A glass "phantom" containing suitable quantities of inorganic phosphate (P_i) was used to calibrate the standard and to provide accurate quantitation in subsequent experiments with the kidney. The "phantom" was a kidney–shaped glass container filled with solutions of 0.2 to 5.0 μmoles P_i. Linearity was good over this range [5]. Calibration was carried out both "in vitro", and with the rat in situ within the magnet bore, the phantom taking the place of the rat's kidney. B_1 homogeneity was therefore close or identical to that used in the in vivo study. The error of repeated assay was ±7% [5], but error due to the effect of the true kidney is very hard to quantify. The relative position of kidney and standard was constant in respect of B_1 field during a single experiment and between separate experiments. The "error" of this measurement was therefore included in the SEM for ATP (about 10%).

NMR spectrometer

The spectrometer used for these studies was a 4.3 Tesla superconducting magnet of 10 cm bore, built in the Biochemistry Department, University of Oxford, and used in previous studies [2]. Of necessity, the animal was held in a vertical position in all studies using this instrument. At the start of the experiment, the homogeneity of the static magnetic field (B_0) was adjusted, using the proton signals of the kidney ('shimming'). This proton frequency also served as a reference, in the absence of PCr, for the determination of pH from the chemical shift of P_i. Qualitative ^{31}P NMR spectra were obtained using a one second delay between pulses. In four animals, T_1 relaxation times for the key phosphorus metabolites of rat kidney in vivo were determined using the method of saturation recovery [13]. Fully quantitative phosphorus spectra of the kidney in vivo can be obtained using pulse intervals $5 \times T_1$ seconds as previously described for the perfused rat kidney [5]. In all spectra reported in the present study, pulse intervals of 12 seconds were used, and collections of 200 to 400 scans made.

Saturation transfer

For the saturation transfer experiment, a second irradiating resonance at the frequency of γ-phosphate of ATP (approximately 280 Hz) was applied [5] and the effect on the magnitude of the P_i resonance was recorded (Ma/Mo where Mo = area of P_i peak during control experiment, and Ma = area of P_i peak during saturation of γ-ATP).

The pulse sequence used was identical to that employed previously [5].

Because there was the possibility of stray radiation altering peak area during saturation, the control spectra were collected while a second irradiation was applied at a frequency equidistant down–field from the P_i frequency (~800 Hz) at 1350 Hz [5]. Finally, random fluctuations in renal [P_i], indicating changes in the amplitude of P_i signal over periods of several min during the course of an experiment, have been observed (unpublished work in this Laboratory) which would significantly alter the observed Ma/Mo. While the time scale is too long to have any effect upon the saturation and transfer of magnetization, changes in [P_i] due to this random fluctuation were corrected by collecting the "control" and saturated spectra alternately, every 20 scans. Spectra were then added sequentially in the computer until 200 or 300 scans for each were obtained. Since each saturation transfer measurement thus depended upon the summation of some 400 to 600 scans, with 12 sec delay, a minimum of 120 min was required. In practice, experiments lasted up to four hours. Stability of the animal during this prolonged period was established from continuous record of the arterial pressure. Experiments on animals in which arterial blood pressure fell below 110 mm Hg were discontinued. In addition, ^{31}P spectra were obtained at the beginning and the end of each study and were comparable.

Calculation of metabolic rate

Metabolic rate is the rate of ATP synthesis, principally the flux through oxidative phosphorylation.

$$ADP + P_i \underset{K_b}{\overset{K_a}{\rightleftharpoons}} ATP$$

The flux (F) = $K_a \times [P_i]$, where K_a is the forward rate–constant and [P_i] the concentration of free–phosphate. This was calculated according to the method of Forsen and Hoffman [9], discussed in a previous paper [5]. The calculation assumes complete reversibility of the reactions involved in ATP synthesis and degradation. Ma/Mo was determined in the saturation transfer experiment: T_{1eff}, the T_1 relaxation time of P_i, was determined in the presence of saturation of γ-ATP, and permitted calculation of both T_{1A} and K_a from the equations:

$$\text{Equation 1:} \quad Ma/Mo = \frac{1}{1 + T_{1A} \cdot K_a}$$

$$\text{Equation 2:} \quad 1 \times \frac{1}{T_{1eff}} = \frac{1}{T_{1A}} + K_a$$

Since one of the aims of this study was to validate the technique of STNMR applied to an intact organ, the relationship between Ma/Mo and flux (F) or other measurements was calculated and linear regression lines plotted.

Enzymatic and chemical analyses

For comparison with NMR-derived values for adenine nucleotides and P_i, kidneys were freeze–clamped in vivo or during isolated perfusion, and assays carried out on perchloric acid extracts, as previously described [5].

Determination of renal blood flow

The method of McDevitt and Nies [14] was followed in detail. This method employs microsphere injection and permits determination of cardiac output and renal blood flow in the same animal. Rats were anesthetised with Inactin (Bayer) 100 mg/100 g body wt. Tracheostomy was performed, and cannula placed in femoral artery, femoral vein and in the dorsal tail vein. Blood pressure was recorded from a transducer via a catheter in the carotid artery. Microspheres, radioactively labelled with either Sc^{46} or St^{85} and 15 μ in diameter, were injected and sampled as described [14]. Organ blood flow was calculated according to standard formula. Experiments in which radioactive counts in left and right kidneys differing by more than 5% were rejected.

In subsequent experiments, renal blood flow was found not to be significantly altered by the use of Nembutal for anesthesia in place of Inactin (Yahaya; unpublished).

Renal oxygen consumption

Using the same conditions as described for the determination of renal blood flow, 0.2 ml samples of blood were carefully and slowly withdrawn from the femoral artery and right renal vein by direct puncture. Haemoglobin, O_2, CO_2 and pH were determined in duplicate, using an EIL automated blood–gas analyser. From H-O_2 dissociation curves of rats, oxygen content was determined. Oxygen consumption for a single kidney was then calculated from the relationship:

$$\frac{\text{(arterial—venous) } O_2 \times \text{flow rate}}{\text{wt of kidney}}$$

Calculation and expression of results

Results for metabolite concentrations were expressed per g dry wt, to correct for the differing water content of normal and perfused kidney, giving mean ± SEM. Student's *t*-test was used for comparisons.

Oxygen consumption, blood flow and metabolic rate were expressed in terms of wet wt of kidney unless otherwise indicated. The ratio of wet wt to dry wt, 3.45 for the kidney in vivo and 5.0 for the perfused kidney, was used as necessary.

ATP:O ratio

The link between oxygen consumption and ATP synthesis by oxidative phosphorylation in tightly coupled mitochondria is expressed by the ratio ATP synthesis to μ atoms oxygen consumed, and is on theoretical grounds between 2 and 4 [15]. The key calculation of ATP:O ratio for the rat kidney in vivo was derived from mean values for STNMR metabolic rate (ATP synthesis: ADP + $P_i \rightarrow$ ATP) and oxygen consumption μ atoms O/min/g kidney, determined as above from a-v O_2 × renal blood flow rate. As already described above, different animals were used for each of these independent measurements. In consequence, a mean value for ATP:O has been obtained, and the error calculated from the individual SEMs.

Problems of quantitation of Fourier Transformed spectra of kidney

Special problems exist in the quantitative treatment of NMR spectra, and additional problems have also been encountered in the present study on the kidney. Solutions to these problems have been applied as follows.

Effects of signal saturation. To permit magnetization to relax back to equilibrium and therefore to obtain the true signal intensity, T_1 relaxation time was determined for each of the metabolites observed in the renal spectrum, and pulse intervals of 5 × T_1 were used throughout. T_1 was determined using saturation recovery [13] method in four kidneys in vivo, using the same preparation as that described above. The ideal of determining T_1 for each kidney before either quantitation or saturation transfer studies could not be achieved, due to the very lengthy nature of these experiments. Viability of the kidney could not be guaranteed over about four to six hours.

Analysis of line–shapes. In order to make quantitative determinations of the amount of metabolites present in an NMR spectrum, a constant method for the analysis of line–shape was required. The problem in renal ^{31}P NMR spectra is that of the overlap between peaks of metabolites with similar resonant frequencies. Peak areas were analyzed ("cut and weigh") by plotting expanded traces of relevant peaks, which produced a result similar in practice to that of the computer integration of the same peak area. Renal spectra contain a broad convolution, thought to be due to phospholipids. The intensities of peaks on top of this "hump" were determined after subtraction of a "hump," which was constructed by eye, for each spectrum. The accuracy of this type of measurement was confirmed by repeated analysis of spectra (± 5% error) and by comparison with chemical assays. Additional difficulties arise when one metabolite (P_i) is found for which only a proportion is visible to NMR. In the saturation–transfer experiment, where a change in the area of P_i signal is sought, errors are likely to be considerable. Part of the objective in the present paper was to estimate the extent of this error by comparison of the saturation–transfer result with independently determined oxygen consumption.

Heterogeneity of the kidney. As is well recognized, NMR signals arising within the volume of the coil are summed, and the resultant signal is the average of all metabolite concentrations of the very heterogeneous cells of the kidney. The assumption is therefore made that ATP and P_i within individual cells are in chemical equilibrium, even if concentration gradients exist between different cells. In the overall determination of metabolic rate by STNMR, it is sufficient to assume that ATP-generating and ATP-consuming reactions are at near equilibrium, and assumption supported by the relative constancy of total renal [ATP].

Measurement of exchange rate with STNMR

The interconversion of two chemical species in equilibrium can be measured by NMR, provided that the rate is of the same order of magnitude as the T_1 rates of the observed nuclei. Using a double resonance technique to saturate the γ-phosphorus of ATP, the change in area of P_i (Ma/Mo) was determined, as discussed immediately above. The remaining unknown in Equations 1 and 2, T_{1A} was accurately determined in four kidneys during saturation of γ-ATP, and this value was then used for the calculations of K in each additional saturation transfer experiment. As with the determination of T_1, the ideal would be the determination of T_{1eff} and T_{1A} for every kidney, but the limits of viability of the kidneys precluded this. Such errors are also included in the final computation of the rate of ATP synthesis contained in this paper, but are likely to be very small [5].

Results

Quantitative ^{31}P spectrum of rat kidney in vivo

Figure 2 is a representative quantitative spectrum of rat kidney in vivo. It was obtained from an indwelling solenoid coil. Acquisitions were Fourier transformed to obtain this spectrum. It is comparable to the qualitative ^{31}P NMR spectra obtained previously in this Laboratory [4] and to the quantitative ^{31}P spectra obtained from the isolated perfused rat kidney [5]. The relative amounts of the six peaks which characterize the kidney spectrum—AMP, inorganic phosphate (P_i), glycerophosphoryl-

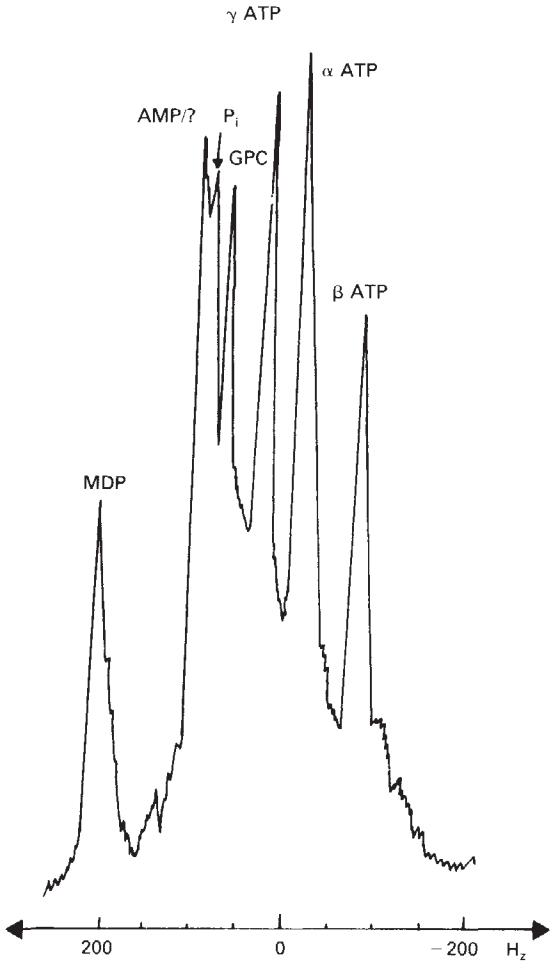

Fig. 2. *600 scans with 12 second delay were collected from an indwelling solenoid coil around the kidney of an anesthetized rat.* The peaks observed were, from left to right: Methylene diphosphonate (4.5 μmoles standard), AMP (plus sugar phosphates and unidentified compound), inorganic phosphate (P_i, glycerophosphoryl choline (GPC), phosphocreatine (PCr), γ-phosphate ATP, α-phosphate ATP (including ADP and pyridine nucleotides), β-phosphate of ATP. The absolute concentrations of these metabolites are given in Table 1.

choline, and the three resonances of ATP—γ, α and β-phosphate—were similar between in vivo and perfused kidneys. Additional peaks attributed to 2:3 diphosphoglyceraldehyde, present in red cells, were not observed in in vivo spectra of the kidney. This may be because the amount of blood and hence of 2:3 diphosphoglyceraldehyde in the intact kidney was insufficient; A further possible explanation relates to the very rapid blood flow through the kidney such that complete exchange may occur every two seconds, a time which is well within the pulse intervals employed for the present study (c 12 secs). Further studies are required to resolve this point. When adequate precautions were taken to exclude signal from adjacent skeletal muscle, no significant amounts of PCr were observed in vivo. The quantity of PCr in intact kidney was therefore below the limit of detection with the present method. As has already been reported [5], there are major differences between the quantity of phosphorus metabolites determined by ^{31}P NMR and by enzymatic analysis of extracts. These differences are

Table 1. Comparison of phosphorus metabolites in vivo and in isolated perfused kidney by ^{31}P NMR.

Rats	Isolated perfused kidney	*P*	Kidney in vivo
ATP (β)	8.4 ± 0.9 (14)	NS	9.1 ± 2.1 (9)
ADP	1.7 ± 0.7 (14)	<0.05	0.03 ± 0.03 (9)
P_i	3.3 ± 0.8 (13)	NS	2.3 ± 0.2 (9)
GPC	8.0 ± 1.2 (11)	<0.05	5.0 ± 0.1 (9)
AMP/?	9.9 ± 1.4 (11)	<0.05	6.4 ± 0.1 (9)

Table 2. Comparison of ^{31}P NMR quantitation in vivo and enzymatic assay of kidney extracts.

Rats	Renal extracts enzymatic assay (*N* = 4)	*P*	^{31}P NMR in vivo (*N* = 9)
ATP	5.8 ± 0.4	NS	9.1 ± 2.1
ADP	2.5 ± 0.2	<0.001	0.07 ± 0.07
AMP	0.4 ± 0.05	<0.001	6.4 ± 0.12
P_i	9.0 ± 1.0	<0.01	2.33 ± 0.2

also consistently found in the intact kidney in vivo. However, there are also some minor differences in detailed comparison of quantitation of metabolites between the kidney in vivo and the isolated perfused kidney (Table 1). ATP content was identical, reflecting the efficient oxygenation of the isolated kidney preparation. But while a significant ADP content was observable by NMR in the isolated kidney, no ADP was observed in the kidney in vivo. Thus, the difference in peak area between β and γATP was 0.03 μmoles ± 0.03, not significantly different from zero. [P_i] was also 30% lower in vivo than in the perfused kidney, but this difference was not statistically significant. Overall, these differences between the kidney in vivo and the isolated perfused kidney result in a significantly higher phosphorylation potential for the in vivo kidney:

$$\frac{[ATP]}{[ADP][P_i]}$$

This in turn may have important consequences for the calculation of free-energy of ATP hydrolysis available for Na^+ transport in vivo.

As expected [5], important differences were also observed between the phosphate metabolite content determined by NMR and by conventional enzymatic analysis, after freeze-clamping of the kidney in vivo (Table 2).

The major differences concerned the large content of ADP and P_i in the kidney which is invisible to NMR, a phenomenon observed and discussed in detail in relation to the perfused kidney [5] and rat kidney extracts [6]. Calculation (not presented in detail) of the possible contribution of urine P_i to the whole signal suggests this to be less than 8% of the total renal P_i that was chemically determined. The significantly greater amount of 'AMP' observed by NMR than by specific enzymatic assay (6.4 μmoles vs. 0.4 μmoles by enzyme assay) reflects an unidentified phosphorus resonance at similar frequency to AMP present in the kidney. Systematic enzymatic degradation of AMP in renal extracts [3, 5] leaves another stable phosphate.

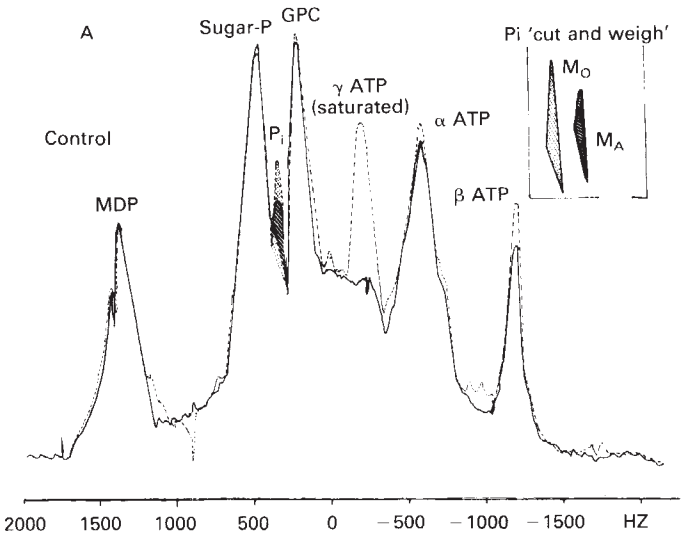

Fig. 3. *Effect of saturating γ-phosphate of ATP of the kidney on $_{31}P$ NMR spectrum in normal rats.* Animals were prepared as described in **Methods** and ^{31}P NMR spectra collected under control conditions and with an irradiating frequency at 280 Hz to saturate the signal of γ-ATP. Summed control spectra and "saturated" spectra from a single experiment have been superimposed and the difference in areas shaded. Apart from saturation, which eliminated most of the resonance of γ-ATP, only P_i was systematically altered in amplitude. From the ratio of areas of the P_i resonance under saturating conditions (Ma) and control (Mo) conditions, flux through ATP synthase could be calculated [5]. These areas, illustrating the method of "cut and weigh", are shown in the inset.

Saturation transfer: evidence for renal ATP synthesis in vivo

Saturating irradiation at the frequency of γ-phosphate of ATP resulted in the complete loss of this signal. In addition, a reduction of magnetisation of P_i was readily observed. This is the phenomenon described as "saturation–transfer", described in detail for the intact kidney in vivo [10] and in a single experiment by Koretsky et al (16). A representative experiment is presented in Figure 3. The two spectra superimposed are the control frequency, and the experimental, in which the irradiating frequency was that coinciding with the γ-phosphorus of ATP. Dark shading represents the differences in area of the γ-phosphorus and the P_i peaks. Small decreases in magnetization of α- and β-phosphorus of ATP were also observed in this experiment, but in the series of experiments which follows, these latter changes were not significantly different from zero. A similar small but insignificant decrease in magnetization of the β-phosphorus of ATP, but not the α-phosphorus, during irradiation of γ-phosphorus has been observed in experiments with the perfused kidney [5]. It was attributed to the Nuclear Overhauser Effect (N.O.E.) [5], and was small compared to the transfer of magnetization from γ-ATP to P_i.

The details of eight consecutive experiments in which the saturation transfer experiment was completed, as described in **Methods**, are presented in Table 3. In all experiments, net transfer of magnetization to P_i was observed, although in one (Expt 56) this was probably not significantly different from the control period. This finding is consistent with the occurrence of ATP synthesis measured by ^{31}P STNMR in eight normal kidneys at a rate of 8.86 ± 1.98 μmoles/min/g dry wt of kidney. Ma/Mo was highly significantly reduced from unity 0.67 ± 0.07 and in vivo was not different from that observed in the perfused rat kidney (0.58 ± 0.08) [5]. However, since $[P_i]$ of the kidney in perfusion was higher the rate of metabolism of the perfused kidney (12.5 ± 2.5 (10)) [5] was somewhat higher than that found in vivo. The difference in rate may be due to one or both of the following: the perfused kidney was supplied with metabolic substrates in great excess, and perfused at a rate ten times higher than the kidney in vivo. Both of these may have contributed to an increase in renal oxygen consumption in the perfused organ. Additionally, and possibly more important, anesthetized rats have a reduced renal blood flow. In the present experiments with the animals anesthetized in vertical posture for three to four hours, renal metabolic rate might well be reduced secondary to a fall in effective renal blood flow. This might be overcome in future by the use of horizontal, wide–bore magnets [17, 18].

A relatively wide scatter of 'normal' values for ATP synthesis in vivo was obtained. This did not correlate with [ATP] determined in the same kidneys in a positive way. Indeed, there was a statistically significant fall in measured [ATP] with increasing ATP-synthesis rate (slope −0.668. $P < 0.05$). We have no explanation for this at present.

We may conclude from the present experiments that saturation transfer NMR can detect ATP synthesis in the kidney in vivo at a rate significantly different from zero, and close to that expected from studies in isolated kidneys.

Renal blood flow and oxygen consumption

The best test of the validity of these determinations of ATP synthesis with STNMR is to compare the measured oxygen consumption of the kidney in vivo with the rate of ATP synthesis. In the isolated perfused kidney, a ratio of ATP:O of 2.54 was determined [5]. Since in vivo the ATP synthesized per atom of oxygen is theoretically considered to lie within the limits of 2 and 3 (15), depending upon the respiratory substrate offered for isolated mitochondria, this relationship should exist for the two measurements in the intact kidney.

In a series of animals anesthetised with Inactin, measurement of renal blood flow using injected microspheres resulted in a value for renal blood flow of 6.72 ± 0.42 ml/min/kidney ($N = 8$). This is equivalent to 4.38 ml/min/g wet wt of kidney, and lies close to previously published values for the anesthetised rat.

Arteriovenous oxygen measurements made in 11 (different) normal animals was 6.55 ± 0.77 KPa, oxygen consumption was 2.57 ± 0.31 μmoles/min/g wet wt. This value is close to the oxygen consumption determined in this Laboratory for the isolated perfused rat kidney (3.3 μmoles/min/g wet wt) [5]. The ATP:O ratio in vivo was therefore 8.86 ± 1.98 (8)/2.57 ± 0.31 (11) × 1/2 = 1.72 ± 0.38 (8). This result is significantly different from 1 and 3. Allowing for the considerable scatter of results, both in the determination of saturation transfer rate (error 22%) and in renal oxygen consumption and blood flow, this value can be recalculated after eliminating values which differ by more than 2 × SEM from the mean to be ATP:O = 2.03 ± 0.14 (3), identical to a ratio of 2.0. This result lies within the theoretical limits expected for isolated renal mitochondria, and indicates that there is tight–coupling of oxidative phosphorylation in the intact kidney in vivo.

Table 3. ^{31}P NMR parameters of normotensive rat kidney in vivo.

Parameter	36	38	55	56	57	59	61	62	mean ± SEM
Wet wt, *g*	0.9	0.96	1.3	1.2	0.96	1.1	1.13	1.13	1.09 ± 0.05
Ma/Mo	0.44	0.54	0.61	0.97	0.41	0.88	0.65	0.82	0.67 ± 0.07
Ka, s^{-1}	0.35	0.30	0.26	0.02	0.41	0.08	0.23	0.12	0.22 ± 0.05
T_{1A}, *s*	3.65	2.84	2.52	1.59	3.42	1.74	2.36	1.86	2.49 ± 0.27
Flux, M^{-1}	16.1	9.56	15.17	0.84	11.13	3.56	10.7	3.79	8.86 ± 1.98
[ATP], *μmoles/g wet*	2.28	1.74	2.05	3.28	1.51	2.39	2.15	3.76	2.36 ± 0.25
[ADP]	—	—	0.24	—	—	—	0.04	—	0.03 ± 0.03
[P_i]	0.78	0.53	0.77	0.63	0.99	0.78	0.73	0.54	0.72 ± 0.05
ATP/ADP × P_i	—	—	11.25	—	—	—	78.4	—	
AMP/?, *μmoles/g wet*	2.19	2.02	1.96	2.02	1.18	2.03	0.99	0.96	1.9 ± 0.04
GPC, *μmoles/g wet*	1.69	2.74	1.29	1.07	0.98	1.87	0.69	6.37	1.5 ± 0.03
pH	7.05	7.31	7.38	7.40	7.18	6.90	7.09	7.21	7.2 ± 0.1
BP *mm Hg*	120	120	150	150	140	170	160	155	146 ± 4

Discussion

The present results confirm and extend the studies with quantitative ^{31}P NMR in the isolated kidney to the kidney in vivo. ATP is virtually all 'visible' to NMR, so that quantitation by this non-destructive technique gives results which are indistinguishable from those obtained by the best freeze–clamping and enzymatic analyses. No systematic error in ATP analysis using the older techniques has been detected, so that for kidney at least, one may be confident that simple extraction into ice–cold perchloric acid is a reliable means of determining this metabolite. Nevertheless, the advantages of so doing without extraction are clear. ^{31}P NMR has an important role in future quantitative analyses in vivo.

As in the isolated kidney, the position is different for ADP and P_i, both of which give very different results by quantitative NMR from the enzymatic assay of extracts. In vivo, virtually no ADP could be detected in the kidney; this is different from the isolated kidney in which a statistically significant proportion, up to 25%, of ADP was observed with NMR. This may have reflected the deficiency in oxygenation of the perfused kidney detected by other techniques [19]. Parallel observations in several other tissues [20] also indicate that the majority of ADP is bound, probably to actin or myosin, and is therefore not visualized with NMR. In the kidney the binding protein, if it is such, is unknown. More detailed analysis of this problem has been presented elsewhere [6]. The important conclusion which can be drawn from these experiments with parallel NMR and enzymatic analysis is that ADP found in extracts cannot simply be there as a result of ischemic breakdown of ATP during tissue extraction.

Similar arguments may be applied to P_i, which in vivo is visible to NMR to the extent of only 26% of the total determined chemically. Urine P_i, which not only comprises less than 8% of total renal P_i, but is also said to have a different chemical shift [16], cannot materially alter this conclusion. This is the same result as was obtained in the isolated kidney, and confirms that the surprisingly low P_i content described is also a property of the renal cell in vivo. For reasons discussed previously [5], the NMR visible P_i (0.7 mM) is considered to be that which is 'free' within the cytoplasm of the cell. The implications of this finding for an understanding of renal P_i transport remain to be fully explored.

Free–energy of ATP hydrolysis

One consequence of these new estimates of ADP and P_i is a reappraisal of the free–energy of ATP hydrolysis ($\Delta G'_{ATP}$). Since this value is proportional to phosphorylation potential:

$$\Delta G' = \Delta G_0' + \frac{ATP}{ADP \times P_i}$$ [5], a value even

higher than that recently reported for the intact perfused rat kidney may obtain in vivo. We have already suggested that this may offer an alternative explanation for the control of sodium transport in the kidney by a pump which is not committed to a mechanism whereby exactly 3 Na may be transported per ATP hydrolized [5]. Instead, the greatly increased free–energy available from each ATP hydrolized may be harnessed to a pump which actively transports sodium against a gradient with similar energy expenditure. Such a pump might be capable of transporting 8 to 12 atoms of sodium per ATP, at the phosphorylation potential now determined for the rat kidney in vivo.

Saturation transfer and the measurement of renal metabolic rate

It seems a reasonable assumption [5] that the quantity of a metabolite which is detected by NMR is that which is free to engage in chemical exchange. A further test of this assumption is provided by the saturation transfer experiment.

Thus, the term [P_i] enters the calculation directly. In practice, the majority of experiments clearly favor the use of the NMR-observed [P_i] in the calculation of the rate of ATP synthesis, giving a value for the kidney in vivo which is within the range of observed renal oxygen consumption [5].

The rate of ATP synthesis in vivo appeared to be somewhat lower than that found for the isolated perfused kidney [5], 8.9 vs. 12.5 μmoles ATP/min/g. It appears likely that under the conditions of these studies, renal metbolic rate was indeed lower in vivo. Thus, animals were anesthetized for prolonged periods in a vertical position. In the isolated perfusion studies on the other hand, artifically high "blood" flow rates and unphysiological substrates were used to maintain viability. The ATP:O ratio of 2.5 reflects this. In the present study, when the results obtained with STNMR in vivo are compared with oxygen consumption measured in different animals (a necessity in the design of the present studies), then an ATP:O ratio of 1.7

results. Given the 20% error probable in both STNMR assay and in renal oxygen consumption determination, based as they are upon renal blood flow measurement, this figure is not sufficiently different from an ATP:O ratio of 2. The kidney in vivo is largely consuming fatty acid as a respiratory fuel [21, 22] with which fuel the ATP:O ratio 2.0 is expected.

Initial enthusiasm for STNMR as a novel tool with which to measure the rates of turnover of metabolites in intact tissues has been modified as theoretical objections have been considered. Thus, STNMR depends upon fully reversible reactions, and reactants for which the fate is predominantly that of the reaction under study. Alternative pathways, or multiple metabolic pools of substrates, would invalidate this simple NMR approach. Other methods, such as 2D NMR, are being explored as alternatives [23]. However, the earlier studies with STNMR in vivo have been largely uncontrolled by comparison with alternative quantitative assays [8]. The present study, in which renal oxygen consumption complies reasonably well with the result obtained with STNMR, goes some way to validating the quantitative NMR assay, and the assumptions that NMR 'visible' pools are synonymous with metabolic activity. It seems reasonable to conclude that with STNMR we should be able to monitor renal ATP synthesis continuously in vivo, and determine the effects of pathological maneuvers upon this rate. A study of the effects of hypotension and renal hypoxia on ATP synthesis has been completed and is described in a succeeding paper [24].

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